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**SUMMARY** The positional and fatty acid specificities of lipases from several different microorganisms have been examined by gas-liquid chromatographic analysis of the free fatty acids following action on mixed triglycerides of known composition. Three types of lipases were found. Lipases of the first type, obtained from several microorganisms, attacked primarily the 1-position of the triglyceride in a manner similar to that of pancreatic lipase. Some fatty acid preference was also observed. The ability of the lipolytic enzymes from *Staphylococcus aureus* and *Aspergillus flavus* to attack the 2-position as well as the 1-position suggested another type of activity. The lack of any appreciable effect of diisopropylfluorophosphate in  $10^{-4}$  M concentrations on this hydrolysis indicated it was not an esterase. The third type of lipase was from *Geotrichum candidum* and it preferentially hydrolyzed the oleate ester from the triglyceride regardless of its position. Possible applications of these lipases are discussed.

**T**HE DEGRADATION of fats by lipases of animal, plant, and microbial origin has been recognized for many years, yet only recently has their activity in terms of types and location of the fatty acids released been investigated. Mattson and Beck (1) and Savary and Desnuelle (2) were the first to show that the site of action of pancreatic lipase was primarily on the terminal fatty acids. The specificity of pancreatic lipase has been reviewed by Desnuelle and Savary (3).

Most of the attempts to compare microbial enzymes of different origin have involved determination of the relative rates of hydrolysis of pure triglycerides (4-6). Known differences in the solubility, ease of emulsification, and stability of emulsions of the various triglycerides limit the value of such comparisons. Richards and El-Sadek, in a study of microbial action on butterfat (7), separated the liberated fatty acids into broad groups on the basis of volatility, and whether solid or liquid. They found some differences in the relative amounts of

these groups that were released by certain bacteria and molds. Wilcox et al. (8), by the use of paper chromatography on steam distillates of butterfat that had undergone microbial lipolysis, found differences in specificity among the various microorganisms.

Studies in our laboratory have been carried out on the production and activity of lipase from *Pseudomonas fragi*, the fatty acids liberated being determined by gas-liquid chromatography (GLC) (9). These studies indicated that this lipolytic activity was similar to pancreatic lipase in that it attacked primarily the 1- (or 3-) position of the triglyceride. Subsequent investigations indicated that different microorganisms might have different patterns of lipolytic activity (10). The present investigation was undertaken, therefore, to explore more thoroughly the indication that lipolysis produced by microbial lipases differed either in position or fatty acid specificity from that shown for pancreatic lipase.

## MATERIALS AND METHODS

Numerous species of microorganisms have been reported to produce lipolytic enzymes. The lipolytic ability of two or more strains of the following species that were known to be lipolytic was studied: *Pseudomonas fragi*, *Pseudomonas fluorescens*, *Staphylococcus aureus*, *Candida lipolytica*, *Geotrichum candidum*, *Penicillium roqueforti*, *Aspergillus flavus*, *Rhizopus oligosporus*, and *Aspergillus niger*. In addition, 73 other cultures of molds and yeast were screened for lipolytic activity.

### *Preliminary Screening of Lipolytic Cultures*

Although an intracellular lipase has been shown to be present in the mycelium of a few fungi (6), extracellular lipases are more common and were the only enzymes examined in this investigation. Batches of various media (described below) were adjusted to pH 5 and 7, inocu-

lated, and incubated at 15°, 20°, 25°, or 30° for intervals of 3–7 days. The cells were removed by filtration or centrifugation and the filtrate or supernatant solution assayed for lipolytic activity as previously described (11). Lipase was produced by molds in medium 2 and in the Czapek's solution recommended by Morris and Jezeski (12). The yeasts were grown in medium 2, and *Candida lipolytica* was grown in media 1 and 4. The bacteria were grown in media 1 and 3. If 2 ml of a culture supernatant solution or filtrate released a net titratable acidity of over 1.0 ml of 0.02 N acid in 1 hr from lard, corn oil, or coconut oil, it was studied further to determine whether changes in cultural conditions would improve lipase production. It should be noted that many cultures showing lipolysis on Nile blue sulfate plates or similar qualitative test media may produce relatively small amounts of extracellular lipase, and these quantities are not readily measurable by titration.

#### *Media for Lipase Production*

The media used to grow the organisms for lipase production were as follows. *Medium 1*, Case peptone 1.0%, 1 M phosphate buffer 5% (pH of buffer 5.0 or 7.0 depending on the culture). *Medium 2*, Case peptone 1.0%, 1 M phosphate buffer 5.0%, yeast extract 0.1% (buffer pH 5.0 and 7.0). *Medium 3*, BBL Trypticase 1.0%, 1 M phosphate buffer 5.0%. *Medium 4*, The *Mycotorula lipolytica* medium of Peters and Nelson (13). *Medium 5*, synthetic medium for *P. fragi* (11).

#### *Preparation of Lyophilized Enzymes*

In order to have a stable enzyme preparation to use for all the assays on the known triglycerides, 2-liter batches of supernatant solution or filtrate containing the enzyme were prepared and dialyzed for 18–24 hr against distilled water. The dialyzed enzyme was concentrated to approximately 5–10% of its original volume by dialyzing against polyethylene glycol (14) at 1–3° overnight. The concentrated enzyme solution was lyophilized and the dry powder stored in a dry ice chest. No change in hydrolytic activity of these preparations was observed after storage for 8 months.

#### *Assay Procedure*

Because of the small quantities of the synthetic triglycerides available for study, it was not possible to prepare emulsions by homogenization as was done for the natural fats and oils (11). These triglycerides (see acknowledgments preceding References for sources) were described as at least 97% pure and only minute traces of free fatty acids could be detected by GLC. Enzymatic hydrolysis of these triglycerides of known composition was determined as follows. Fifty milligrams of the triglyceride was weighed into a 20 ml sonication tube and 2 ml of 0.25

M phosphate buffer at the pH indicated in Table 1, 0.3 ml of 1%  $\text{CaCl}_2$ , 0.1 ml of 2.5% Astec 4135 (an emulsifier from Associated Concentrates, Inc., Woodside, N. Y.),<sup>1</sup> and 5.5 ml of water were added. This tube was held at 55–60° until the triglyceride melted. (For saturated triglycerides such as 2-palmito-distearin a temperature nearer 70° was required.) The mixture was then sonicated on a 60w, 20 kc/sec MSE ultrasonic disintegrator (Instrumentation Associates, Inc., New York, N. Y.) at maximum amperage for 4 min with the temperature maintained at 55–60°. The emulsion was immediately transferred to a Mojonnier flask and placed in a water bath at 35°. Within 10 min the temperature of the emulsion had fallen to 35° and 2 ml of enzyme solution was added. (The lyophilized enzyme was used in a concentration of 2 mg/ml.) After incubation without agitation for 1–3 hr the mixture was acidified, the fat and free fatty acids were extracted twice with petroleum ether, and the free fatty acids separated from the glycerides on an ion-exchange resin. The free fatty acids were converted to methyl esters and their composition determined on a Beckman GC-2 gas chromatograph equipped with a thermal conductivity detector as previously described (15), except that 20% diethylene glycol succinate on Chromosorb P was used to pack the column in the latter part of the investigation. Each enzyme was assayed at least twice on each triglyceride, and in several instances three or more assays were run.

Calcium has been shown to enhance the activity of certain lipases (16), although its mode of action is not definitely known. Preliminary tests with the lyophilized enzymes on the synthetic triglycerides showed that calcium would increase the activity of several of them. It was, therefore, added routinely to all assay flasks.

#### *Effect of Diisopropylfluorophosphate (DFP) on Activity*

DFP has been shown to be an effective inhibitor of esterases, but not of lipases (17). To determine its effect on the enzymes in this study, various concentrations were added to the enzyme, then allowed to stand for 30 min before the treated enzyme was assayed.

## RESULTS

#### *Selection of Microorganisms for Intensive Study*

Of the 82 microorganisms included in the survey, only 13 of them produced lipase of sufficient activity to warrant further study. It is quite possible, of course, that other conditions of culture would have resulted in a larger number of active lipase producers. This is amply supported by the work in developing a medium for

<sup>1</sup> The mention of specific products is for identification only and implies no endorsement of the product.

TABLE 1 MEDIA, GROWTH, AND ASSAY CONDITIONS FOR VARIOUS CULTURES

Culture	Medium	Production			Assay
		Temperature	Time	pH	pH*
			Days		
<i>P. fragi</i>	5	20	4	7.0	7.0
<i>P. fluorescens</i>	3	20	4	7.0	7.0
<i>P. geniculata</i>	1	20	4	7.0	7.0
<i>S. aureus</i>	3	30	4	7.0	7.0
<i>G. candidum</i>	1	20	3	7.0	6.0
<i>C. lipolytica</i>	4	25	4	4.5	5.5
<i>A. flavus</i>	2	25	5	7.0	7.0
<i>P. roqueforti</i>	2	20	7	7.0	7.0
<i>R. oligosporus</i>	2	20	5	6.0	7.0
<i>C. fresenii</i>	1	15	8	7.0	7.0
<i>T. elegans</i>	2	20	6	6.0	6.0
<i>P. nitens</i>	2	25	4	6.0	7.0
<i>M. sufu</i>	2	25	3	7.0	7.0

\* Values optimal for activity of respective enzymes.

maximum production by *P. fragi* (11). None of the yeast organisms was included among the 13, although one strain of *Hansenula ciferrii* produced measurable amounts and will be studied further. The cultures named in Table 1 produced a net titratable acidity of more than 1 ml of 0.02 N acid within 30 min when the substrate was lard, corn oil, or coconut oil. These cultures were selected for investigation of their activity on triglycerides of known structure.

#### Effect of DFP

None of the lipases included in this work except that from *G. candidum* was affected by a  $10^{-4}$  M concentration of DFP and none of them exhibited more than a 25–35% reduction in activity in the presence of  $10^{-3}$  M DFP. *G. candidum* was not affected by  $10^{-5}$  M and showed only a 25–35% reduction by the  $10^{-4}$  M concentration of DFP. Reports in the literature indicate that esterases are inhibited by DFP in concentrations of  $10^{-5}$  M or less, whereas lipases are not affected by these concentrations.

TABLE 2 LIPOLYSIS OF SYNTHETIC TRIGLYCERIDES BY LIPASE FROM *PSEUDOMONAS FRAGI*

Triglyceride	Weight Per Cent of Free Fatty Acids as:		
	Palmitic	Stearic	Oleic
2-Stearoyl diolein		2	98
2-Oleoyle distearin		97	3
2-Palmitoyl diolein			99
2-Oleoyle dipalmitin	96		4
2-Oleoyle palmito-stearin	55	45	
2-Palmitoyl distearin	6	94	
2-Stearoyl dipalmitin	99	1	
1-Oleoyle distearin		33	67
1-Stearoyl diolein		15	85
1-Oleoyle dipalmitin	47		53
1-Palmitoyl diolein	48		52

#### Action of Lipase from *P. fragi*

The types and percentages of fatty acids released from 11 different triglycerides by *P. fragi* are shown in Table 2. In each instance uninoculated controls were included and any free fatty acids found were subtracted from those found in the assay flask. Earlier investigations in our laboratory indicated that the lipase of *P. fragi*, like pancreatic lipase, attacks di- and monoglycerides at a much slower rate than it does triglycerides. If monoglycerides were being attacked, some of the fatty acids esterified at the 2-position of the triglyceride would be present in the hydrolysate. Since the triglycerides investigated contained fatty acids of similar molecular weight, and the assay time was limited to that yielding a weight of free fatty acid less than 30% of the triglyceride, one can be reasonably certain that the action being measured primarily represents action on triglycerides and not on diglycerides.

It is apparent from the results shown in Table 2 for the first six triglycerides that this lipase has very slight, if any, action on the 2-position. If one considers the lower molecular weight of palmitic (mol wt 256) as compared to oleic (mol wt 282) and stearic (mol wt 284) acids, the weight percentages shown in this table indicate that palmitic acid is as readily attacked as oleic (cf. 1-oleoyl dipalmitin and 1-palmitoyl diolein, a molar ratio near 1:1), and that it is more easily attacked than stearic (cf. 2-oleoyl palmito-stearin, a molar ratio near 3:2).

#### Action of Lipase from *S. aureus*

The types and percentages of fatty acids released from the 11 triglycerides by *S. aureus* lipase are shown in Table 3. As with the lipase from *P. fragi*, the total amount of fatty acid released during the assay was less than 15 mg. The results with the first six triglycerides clearly indicate that this lipase can attack the 2-position as well as the 1-position. The data also show that this enzyme has a slight preference for oleic over stearic linkages but the difference is not as great as with the *P. fragi* lipase.

#### Action of Lipase from *G. candidum*

The types and percentages of fatty acids released from the different triglycerides by *G. candidum* lipase are shown in Table 4. This enzyme represents a third type of activity in that it exhibits a decided preference for the unsaturated acid oleic. This preference is evident regardless of whether the oleic is in the 1- or 2-position. As is shown by the data on 2-oleoyl palmito-stearin, 2-palmitoyl distearin, and 2-stearoyl dipalmitin, the palmitic acid linkage is more readily attacked than the stearic, although neither is attacked at a rapid rate.

TABLE 3 LIPOLYSIS OF SYNTHETIC TRIGLYCERIDES BY LIPASE FROM STAPHYLOCOCCUS AUREUS

Triglyceride	Weight Per Cent of Free Fatty Acids as:		
	Palmitic	Stearic	Oleic
2-Stearoyl diolein		25	75
2-Oleoyl distearin		62	38
2-Palmitoyl diolein	30		70
2-Oleoyl dipalmitin	78		22
2-Oleoyl palmito-stearin	49	32	19
2-Palmitoyl distearin	26	74	
2-Stearoyl dipalmitin	75	25	
1-Oleoyl distearin		55	45
1-Stearoyl diolein		24	76
1-Oleoyl dipalmitin	73		27
1-Palmitoyl diolein	30		70

#### Action of Lipases from Other Microorganisms

The ten additional microorganisms which were studied in some detail showed a pattern of activity similar to either *P. fragi* or *S. aureus*. The cultures and the type of action found are given in Table 5. The action of all the lipases was apparently a species characteristic because at least two strains of each culture gave similar patterns. In the case of the *G. candidum* four strains showed the same preference for oleic acid.

## DISCUSSION

In a paper describing a refined method for the determination of fatty acid distribution in triglycerides by the use of pancreatic lipase (18), Mattson and Volpenhein stated that hydrolysis of the primary ester groups is random and shows no fatty acid specificity. Entressangles et al. (19), however, have reported some differences in rate of hydrolysis among fatty acids by this enzyme. With the microbial lipases reported here, definite differences in the rates of hydrolysis were observed. Since this preferential hydrolysis was manifested when the fatty acids were part of the same triglyceride, the physical state of the emulsion

TABLE 4 LIPOLYSIS OF SYNTHETIC TRIGLYCERIDES BY LIPASE FROM GEOTRICHUM CANDIDUM

Triglyceride	Weight Per Cent of Free Fatty Acids as:		
	Palmitic	Stearic	Oleic
2-Stearoyl diolein		1	99
2-Oleoyl distearin		2	98
2-Palmitoyl diolein	1		99
2-Oleoyl dipalmitin	20		80
2-Oleoyl palmito-stearin	9	1	90
2-Palmitoyl distearin	50	50	
2-Stearoyl dipalmitin	99	1	
1-Oleoyl distearin		2	98
1-Stearoyl diolein		1	99
1-Oleoyl dipalmitin	35		65
1-Palmitoyl diolein	5		95

TABLE 5 SUMMARY OF PRINCIPAL TYPE OF LIPOLYTIC ACTIVITY EXHIBITED BY VARIOUS MICROORGANISMS

Attacks primarily the 1-position	<i>Pseudomonas fragi</i> , <i>Pseudomonas fluorescens</i> , <i>Pseudomonas geniculata</i> , <i>Candida lipolytica</i> , <i>Phycomyces nitens</i> , <i>Mucor sufu</i> , <i>Penicillium roqueforti</i> , <i>Rhizopus oligosporus</i> , <i>Chaetostylum fresnei</i> , <i>Thamnidium elegans</i>
Attacks either the 1-, 2-, or 3-position	<i>Staphylococcus aureus</i> , <i>Aspergillus flavus</i>
Attacks oleic acid linkage regardless of position	<i>Geotrichum candidum</i>

should not have been a factor as it would be when comparing simple triglycerides or mixtures of them.

The ability of lipases from *S. aureus* and *A. flavus* to hydrolyze the 2-position at about the same rate as the 1-position suggested a nonspecific attack on ester linkages. However, studies with DFP, fatty acid esters, and a limited number of mono- and diglycerides indicated that they were not simple esterases. Further studies on the residual glycerides, as well as purification and characterization of the enzyme, are needed before this can be ascertained.

The lipase from *G. candidum* is unusual in that it has a high degree of specificity for unsaturated fatty acids; the position of the fatty acid in the molecule has only a secondary effect. This type of activity was unique among the microorganisms studied.

It is apparent from these results that the most prevalent type of lipase present in the microorganisms studied is similar to pancreatic lipase, although there may be greater differences in rate of hydrolysis of certain fatty acids by the microbial lipases than by pancreatic lipase. The high degree of positional specificity of this *P. fragi* type of lipase suggests it could be used for studying the structure of triglycerides in a manner similar to pancreatic lipase (20). Its use at a neutral or slightly acid pH might reduce the acyl migration that occurs at the pH of 8.0 usually employed for pancreatic lipase studies. The lipase from *G. candidum* offers possibilities as a relatively simple means of selectively removing unsaturated fatty acids from a molecule with a minimum effect on both the fatty acid and the remaining portion of the molecule.

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